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(71) Applicants *(for all designated States except US)*: ALBANY MEDICAL COLLEGE [US/US]; 47 New Scotland Avenue, Albany, NY 12208 (US). UNIVERSITY OF MEDICINE AND DENTISTRY OF NEW JERSEY [US/US]; University Heights, 30 Bergen Street, Newark, NJ 07107-3000 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: MANNINO, Raphael, James [US/US]; 22 Victoria Drive, Annandale, NJ 08801 (US). GOULD-FOGERITE, Susan [US/US]; 6 Cynthia Court, Annandale, NJ 08801 (US).

(74) Agents: MACK, Susan, J. et al.; Sughrue, Mion, Zinn, MacPeak & Seas, Suite 800, 2100 Pennsylvania Avenue, N.W., Washington, DC 20037-3202 (US).

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(54) Title: COCHLEATE PHOSPHOLIPIDS IN DRUG DELIVERY

(57) Abstract

The instant disclosure relates to cochleates comprising a) a biologically relevant molecule component, b) a negatively charged lipid component, and c) a divalent cation component. The cochleate has an extended shelf life, even in a desiccated state. Advantageously, the cochleate can be ingested. The biologically relevant molecule can be a polynucleotide or a polypeptide.

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COCHLEATE PHOSPHOLIPIDS IN DRUG DELIVERY

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This is a continuation-in-part of Application Serial No. 08/394170 filed 22 February 1195, which is a continuation-in-part of Application Serial No. 08/130986 filed 4 October 1993.

FIELD OF THE INVENTION

The instant invention relates to cochleates and use thereof to stabilize biologic molecules, such as carbohydrates, vitamins, minerals, polynucleotides, polypeptides, lipids and the like. Cochleates are insoluble stable lipid-divalent cation structures into which is incorporated the biologic molecule. Because cochleates can be biologically compatible, cochleates can be administered to hosts by conventional routes and can serve to deliver the biologic molecule to a targeted site in a host.

BACKGROUND OF THE INVENTION

Plain lipid cochleates (Figure 1) have been described previously. Protein-cochleates or peptide-cochleates have been described heretofore and patented by the instant inventors, as intermediate structures which can be converted to protein-lipid vesicles (proteoliposomes) (Figure 2) by the addition of calcium chelating agents (see U.S. Pat. No. 4,663,161 and U.S. Pat. No. 4,871,488, the disclosures of which expressly are incorporated herein by reference). Freeze-fracture electron micrographs of protein-cochleates containing Sendai glycoproteins made by the DC method show the rolled up lipid bilayer structures with a "bumpy" surface. Plain phospholipid cochleates are smooth in that type of preparation.

The proteoliposomes resulting from polypeptide-cochleates have been shown to be effective immunogens when administered to animals by intraperitoneal and intramuscular routes of immunization (G. Goodman-Snitkoff, et al., J. Immunol., Vol. 147, p.410 (1991); M.D. Miller, et al., J. Exp. Med., Vol. 176, p. 1739 (1992)). Further, when the glycoproteins of Sendai or influenza virus are reconstituted by that method, the proteoliposomes are effective delivery vehicles for encapsulated proteins and DNA to animals and to cells in culture (R.J. Mannino and S. Gould-Fogerite, Biotechniques, Vol. 6, No. 1, pp. 682-690 (1988); S. Gould-Fogerite et al., Gene, Vol. 84, p. 429 (1989); M.D. Miller, et al., J. Exp. Med., Vol. 176, p. 1739 (1992)).

It would be advantageous to provide a means for stabilizing or preserving biologic molecules in a form that is stable at room temperature, capable of desiccation and is suitable for oral administration. For example, it would be beneficial to have a formulation for stabilizing polynucleotides and which could be used for delivering polynucleotides to a cell.

SUMMARY OF THE INVENTION

Accordingly, it is an object of the instant invention to provide a means for stabilizing biologic molecules to yield a formulation with prolonged shelf life, which can be made into powder form and which later can be rehydrated to yield a biologically active molecule.

It also is an object of the instant invention to provide a formulation suitable for use as a vehicle to administer a biologically active molecule to a host. The formulation can be used to

deliver a biologic molecule to the gut for absorption or to a targeted organ, tissue or cell.

A suitable biologic molecule is a polynucleotide.

Other suitable biologic molecules are polypeptides such as hormones and cytokines.

Yet other suitable biologic molecules are bioactive compounds such as drugs.

Those and other objects have been obtained by providing a cochleate formulation comprising the following components:

a) a biologically relevant molecule component to be stabilized or delivered,

b) a negatively charged lipid component, and

c) a divalent cation component.

In a preferred embodiment, the cochleate formulation is administered orally.

The instant invention further provides a cochleate formulation containing a polynucleotide, wherein said polynucleotide-cochleate comprises the following components:

a) a polynucleotide component,

b) a negatively charged lipid component, and

c) a divalent cation component.

The polynucleotide can be one which is expressed to yield a biologically active polypeptide or polynucleotide. Thus, the polypeptide may serve as an immunogen or, for example, have enzymatic activity. The polynucleotide may have catalytic activity, for example, be a ribozyme, or may serve as an inhibitor of transcription or translation, that is, be an antisense molecule. If expressed, the polynucleotide would include the necessary

regulatory elements, such as a promoter, as known in the art.

The instant invention further provides a cochleate formulation containing a polypeptide, wherein said polypeptide-cochleate comprises the following components:

a) a polypeptide component,

b) a negatively charged lipid component, and

c) a divalent cation component.

A specific example is an insulin cochleate.

The advantages of cochleates are numerous. The cochleates have a nonaqueous structure while not having an internal aqueous space, and therefore cochleates:

(a) are more stable than liposomes because the lipids in cochleates are less susceptible to oxidation;

(b) can be stored lyophilized which provides the potential to be stored for long periods of time at room temperatures, which would be advantageous for worldwide shipping and storage prior to administration;

(c) maintain structure even after lyophilization, whereas liposome structures are destroyed by lyophilization;

(d) exhibit efficient incorporation of biological molecules, particularly with hydrophobic moieties into the lipid bilayer of the cochleate structure;

(e) have the potential for slow or timed release of the biologic molecule in vivo as cochleates slowly unwind or otherwise dissociate;

(f) have a lipid bilayer matrix which serves as a carrier and is composed of simple lipids which are found in animal and plant cell membranes, so

that the lipids are non-toxic, non-immunogenic and non-inflammatory;

(g) contain high conc ntration of divalent cation, such as, calcium, an essential mineral;

(h) are safe, the cochleates are non-living subunit formulations, and as a result the cochleates have none of the risks associated with use of live vaccines, or with vectors containing transforming sequences, such as life threatening infections in immunocompromised individuals or reversion to wild type infectivity which poses a danger to even healthy people;

(i) are produced easily and safely; and

(j) can be produced as defined formulations composed of predetermined amounts and ratios of biologically relevant molecules, including polypeptides, carbohydrates and polynucleotides, such as DNA.

The advantages of oral administration also are numerous. An oral route has been chosen by the WHO Children's Vaccine Initiative because of ease of administration. Oral vaccines are less expensive and much safer to administer than parenterally (intramuscular or subcutaneous) administered vaccines. The use of needles adds to the cost, and also, unfortunately, in the field, needles are often reused.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic representation of a plain lipid cochleate.

Figure 2 shows the structure of polypeptide-lipid vesicles with integrated membrane proteins.

Figure 3 summarizes the various alternative procedures for the preparation of cochleates.

Figures 4(A) and 4(B) show serum antibody titers in mice following oral administration of influenza polyp ptide-cochleates.

Figure 5 is a graph showing the results of oral administration of polypeptide-cochleates when challenged with live virus.

Figure 6 is a graphic representation of serum antibody titers in mice following oral administration of Sendai-cochleates.

Figure 7 is a graph depicting the induction of antigen-specific cytotoxic splenocytes following oral administration of Sendai cochleates.

Figure 8 provides a series of bar graphs depicting serum glucose levels before and after oral insulin adiministration.

DETAILED DESCRIPTION OF THE INVENTION

The instant inventors have now found surprisingly and have demonstrated that cochleates themselves be used as means for stabilizing and delivering biologic molecules. The cochleates survive the harsh acid environment of the stomach, protecting the susceptible biologic molecules immersed therein, probably by virtue of their unique multilayered precipitate structure. It is likely that cochleates then are taken up by microfold cells (M cells) in the small intestine.

The instant inventors have demonstrated that oral administration by drinking cochleates containing the glycoproteins and viral lipids from the surface of influenza or Sendai viruses plus phosphatidylserine and cholesterol, stimulate both mucosal and circulating antibody responses. In addition, strong helper cell (proliferative) and killer (cytotoxic) cell responses also are generated. Perhaps most impressively, oral

administration of the influenza cochleates protects against intranasal challenge with live virus.

Those results are unexpected for a number of reasons.

It was not known and was not expected that the cochleates would survive the stomach and protect the polypeptides associated with them from the acid environment and degradative enzymes. It is known that without the presence of at least 3 mM calcium, the cochleates begin to unwind and form liposomes. It was possible, in fact likely, that the cochleates would not remain intact during the transit from the mouth, down the esophagus and through the stomach. If cochleates did come apart, they would be digested as food.

Also, having survived the stomach, that the cochleates would interact in an effective way with the mucosal and circulating immune systems was unknown and unexpected. Everyone ingests large quantities of proteins, fats and sugars on a daily basis which simply get digested and used as fuel, without stimulating any kind of mucosal or circulating immune responses. Thus, the cochleates deliver molecules which retain biologic activity at the delivery site within the host.

As used herein, the term "immune response" means either antibody, cellular, proliferative or cytotoxic activities, or secretion of cytokines.

Also, as used herein, the term "antigen" is meant to indicate the polypeptide to which an immune response is directed or an expressible polynucleotide encoding that polypeptide.

"Polynucleotide" includes DNA or RNA, as well as antisense and enzymatically active molecules. Thus the biologically relevant molecule can be the polynucleotide itself, the transcript thereof or the translated polypeptide encoded thereby.

"Polypeptide" is any oligomer or polymer of amino acids. The amino acids can be L-amino acids or D-amino acids.

A "biologically relevant molecule" is one that has a role in the life processes of a living organism. The molecule may be organic or inorganic, a monomer or a polymer, endogenous to a host organism or not, naturally occurring or synthesized in vitro and the like. Thus, examples include, vitamins, minerals, amino acids, toxins, microbicides, microbistats, co-factors, enzymes, polypeptides, polypeptide aggregates, polynucleotides, lipids, carbohydrates, nucleotides, starches, pigments, fatty acids, hormones, cytokines, viruses, organelles, steroids and other multi-ring structures, saccharides, metals, metabolic poisons, drugs and the like.

The instant invention also can be practiced using whole cells other subcellular replicative entities, such as viruses and viroids. Hence, bacteria, yeasts, cell lines, viruses and the like can be mixed with the relevant lipid solution, caused to precipitate to yield structures wherein the cells and the like are fixed within the cochleate structure.

Polypeptides are suitable molecules to be incorporated with cochleates. The procedure for preparing cochleates is set forth in greater detail hereinbelow. The polypeptide is suspended in a suitable aqueous buffer. The lipids are dried to form a thin film. Then the aqueous buffer is added to the lipid film. The vessel is vortexed and then the sample dialyzed against a cation-containing buffer.

In that way, for example, cochleates carrying insulin can be obtained. The insulin cochleates were made with a 1 mg/ml solution of insulin, but

various other beginning concentrations of insulin can be used to obtain cochleates loaded with varying concentrations of insulin.

Recent studies indicate that the direct injection of DNA plasmids can lead to the expression of the proteins encoded by those plasmids resulting in humoral and cell mediated immune responses, see, for example, Wang et al., Proc. Natl Acad. Sci. 90: 4156-4160 (1993); Zhu et al., Science 261: 209-211 (1993). Those studies indicate that DNA vaccines could provide a safe and effective alternative for human vaccination. Those studies also suggest that DNA vaccines could benefit from simple, more efficient delivery systems.

The use of lipids to facilitate the delivery, entry and expression of DNA in animal cells is well documented, see, for example, Philip et al., Mol. Cell Biol. 14: 2411-2418 (1994). Indeed, DNA-lipid complexes currently form the basis for a number of human gene therapy protocols.

Because cochleates are stable structures which can withstand a variety of physiologic conditions, cochleates are suitable means for delivering biologic molecules, such as, polypeptides or polynucleotides, to a selected site in a host. The polypeptide or polynucleotide is incorporated into and integral with the cochleate structure. Thus the polypeptide or polynucleotide, which may need to be expressed, are protected from degrading proteases and nucleases.

The cochleates used in the instant invention can be prepared by known methods such as those described in U.S. Patent No. 4,663,161, filed 22 April 1985, U.S. Patent No. 4,871,488, filed 13 April 1987, S. Gould-Fogerite et al., Analytical Biochemistry, Vol. 148, pages 15-25 (1985);

S. Gould-Fogerite et al., Advances in Membrane Biochemistry and Bioenergetics, edited by Kim, C.H., Tedeschi, T., Diwan, J.J., and Salerno, J.C., Plenum Press, New York, pages 569-586 (1988); S. Gould-Fogerite et al., Gene, Vol. 84, pages 429-438 (1989); Liposome Technology, 2nd Edition, Vol. I, Liposome Preparation and Related Techniques, Vol. II, Entrapment of Drugs and Other Materials, and Vol. III, Interactions of Liposomes with the Biological Milieu, all edited by Gregory Gregoriadis (CRC Press, Boca Raton, Ann Arbor, London, Tokyo), Chapter 4, pp 69-80, Chapter 10, pp 167-184, and Chapter 17, pp. 261-276 (1993); and R.J. Mannino and S. Gould-Fogerite, Liposome Mediated Gene Transfer, Biotechniques, Vol. 6, No. 1 (1988), pp. 682-690.

The polynucleotide can be one which expresses a polypeptide, that is, pathogen membrane polypeptides, aberrant or atypical cell polypeptides, viral polypeptides and the like, which are known or which are suitable targets for host immune system recognition in the development of immunity thereto.

The polynucleotide may express a polypeptide which is biologically active, such as, an enzyme or structural or housekeeping protein.

Also, the polynucleotide may be one which necessarily is not expressed as a polypeptide but nevertheless exerts a biologic effect. Examples are antisense molecules and RNA's with catalytic activity. Thus, the expressed sequence may on transcription produce an RNA which is complementary to a message which, if inactivated, would negate an undesired phenotype, or produce an RNA which recognizes specific nucleic acid sequences and cleaves same at or about that site and again, the

non-expression of which would negate an undesir d phenotype.

The polynucleotide need not be expressed but may be used as is. Thus, the polynucleotide may be an antisense molecule or a ribozyme. Also, the polynucleotide may be an immunogen.

Thus, for polynucleotides, the relevant coding sequence is subcloned downstream from a suitable promoter, other regulatory sequences can be incorporated as needed, in a vector which is expanded in an appropriate host, practicing methods and using materials known and available in the art.

For example, two plasmids, pDOLHIVenv (AIDS Research and Reference Reagent Program, Jan. 1991 catalog p. 113; Freed et al. J. Virol. 63: 4670 (1989)) and pCMVHIVLenv (Dr. Eric Freed, Laboratory of Molecular Immunology, NJAID, NIH) are suitable expression plasmids for use in polynucleotide-cochleates.

The plasmids contain the open reading frames for the env, tat and rev coding regions of HIV-1 (LAV strain).

pDOLHIVenv was constructed by introducing the SalI-XhoI fragment from the full length infectious molecular clone pNL4-3 into the SalI site of the retrovirus vector, pDOL (Korman et al. Proc. Natl. Acad. Sci. 84: 2150 (1987)). Expression is from the Moloney murine virus LTR.

pCMVHIVLenv was constructed by cloning the same SalI-XhoI fragment into the XhoI site of the cytomegalovirus (CMV)-based expression vector p763.

The polynucleotide can be configured to encode multiple epitopes or epitopes conjugated to a known immunogenic peptide to enhance immune system recognition, particularly if an epitope is only a few amino acids in size.

To form cochleate precipitates, a majority of the lipid present should be negatively charged. One type of lipid can be used or a mixture of lipids can be used. Phosphatidylserine or phosphatidylglycerol generally have been used. Phosphatidylinositol also forms a precipitate which converts to liposomes on contact with EDTA. A substantial proportion of the lipid can, however, be neutral or positively charged. The instant inventors have included up to 40 mol% cholesterol based on total lipid present and routinely make polypeptide-lipid or polynucleotide-lipid cochleates which contain 10 mol% cholesterol and 20% viral membrane lipids. Phosphatidylethanolamine, plain or cross-linked to polypeptides, also can be incorporated into cochleates.

While negatively charged lipid can be used, a negatively charged phospholipid is preferred, and of those phosphatidylserine, phosphatidylinositol, phosphatidic acid and phosphatidylglycerol are most preferred.

One skilled in the art can determine readily how much lipid must be negatively charged by preparing a mixture with known concentrations of negative and non-negative lipids and by any of the procedures described herein, determining whether precipitates form.

There are several known procedures for making the cochleates of the instant invention and those are schematized in Figure 4.

A suitable procedure for making cochleates is one wherein a negatively charged lipid such as phosphatidylserine, phosphatidylinositol, phosphatidic acid or phosphatidylglycerol in the absence or presence of cholesterol (up to 3:1, preferably 9:1 w/w) are utilized to produce a

suspension of multilamellar lipid vesicles containing or surrounded by a biologically relevant molecule (polyp ptide, polysaccharide or polynucleotide, such as DNA) which are converted to small unilamellar protein lipid vesicles by sonication under nitrogen. Alternatively, to avoid damage, the biologically relevant molecule can be added to the solution following sonication. The vesicles are dialyzed at room temperature against buffered divalent cation, e.g., calcium chloride, resulting in the formation of an insoluble precipitate which may be presented in a form referred to as a cochleate cylinder. After centrifugation, the resulting pellet can be taken up in buffer to yield the cochleate solution utilized in the instant invention.

In an alternative and preferred embodiment, an amount of negatively charged lipid, e.g., phosphatidylserine and cholesterol in the same proportions as above and equal to from about 1 to 10 times the weight, preferably equal to four times the weight of the viral or other additional lipids are utilized to prepare the cochleates. Either a polypeptide, mineral, vitamin, carbohydrate or polynucleotide, such as DNA, is added to the solution. That solution then is dialyzed against buffered divalent cation, e.g., calcium chloride, to produce a precipitate which can be called a DC (for direct calcium dialysis) cochleate.

An additional, related method for reconstituting cochleates has been developed and is called the LC method (liposomes before cochleates). The initial steps involving addition of extracted polypeptide, polysaccharide,polynucleotide, such as DNA or combinations thereof, to dried down negatively charged lipid and cholesterol are the same as for the DC method. However, the solution

next is dialyzed against buffer (e.g., 2 mM TES, 2 mM L-histidine, 100 mM NaCl, pH 7.4) to form small liposomes containing the polypeptide, polynucleotide, such as DNA, and/or polysaccharide. A divalent cation, e.g., calcium, then is added either directly or by dialysis to form a precipitate which can consist of cochleates.

In the above procedures for making the cochleates of the instant invention, the divalent cation can be any divalent cation that can induce the formation of a cochleate or other insoluble lipid-antigen structures. Examples of suitable divalent cations include Ca^{+2}, Mg^{+2}, Ba^{+2}, and Zn^{+2} or other elements capable of forming divalent ions or other structures having multiple positive charges capable of chelating and bridging negatively charged lipids.

Cochleates made with different cations have different structures and convert to liposomes at different rates. Because of those structural differences, the rate of release of the biologically relevant molecules contained therewith varies. Accordingly, by combining cochleates made with different cations, formulations which will release the biologically relevant molecule over a protracted period of time are obtainable.

The amount of biologically relevant molecule incorporated into the cochleates can vary. Because of the advantageous properties of cochleates generally, lesser amounts of biologically relevant molecule can be used to achieve the same end result as compared to using known delivery means.

An artisan can determine without undue experimentation the optimal lipid:biologically relevant molecule ratio for the targeted purposes. Various ratios are configured and the progress of precipitation of each sample is monitored visually

under a phase contrast microscope. Precipitation to form, for example, cochleates, is monitored readily. Th n, the precipitates can be administered to the targeted host to ascertain the nature and tenor of the biologic response to the administered cochleates.

It should be evident that the optimized ratio for any one use may range from a high ratio, for example, to minimize the use of a rare biologically relevant molecule, to a low ratio to obtain maximal amount of biologically relevant molecule in the cochleates.

Cochleates can be lyophilized and stored at room temperature indefinitely or can be stored in a divalent cation-containing buffer at 4°C for at least six months.

The cochleate formulations also can be prepared both with and without fusogenic molecules, such as Sendai virus envelope polypeptides. Prior studies with proteoliposomes have demonstrated that cytoplasmic delivery of liposome contents requires a fusogenic liposome bilayer. The exact role of Sendai virus envelope polypeptides in facilitating the immune response to polypeptide-cochleates as yet is not clear.

It is preferred to use cochleates without fusogenic molecules over fusogenic molecule cochleates because of a more simple structure and ease of preparation favors eventual use in humans.

Because polynucleotides are hydrophilic molecules and cochleates are hydrophobic molecules that do not contain an internal aqueous space, it is surprising polynucleotides can be integrated into cochleates. The polynucleotides are not exposed on the surface of the cochleates because the polynucleotides are resistant to nucleases.

In the cas of polynucleotide cochleates, considerations for dosage parallel the standard methodologies regarding vaccines as known in the art. Also, methods for using polynucleotides in liposomes and the "naked DNA" are available to serve as a baseline for empirically determining a suitable dosing regimen, practicing known methods.

For example, a suitable scheme for determining dosing is as follows.

The initial dose of polynucleotides in cochleates administered by injection to animals is selected to be about 50 μg, although it is know that as little as 2μg of tested plasmids is effective. That dose is proposed to maximize the probability of observing a positive response following a single administration of a cochleate. Any formulations which do not elicit a response at that dose are to be considered ineffective but retained for further study.

Developing formulations which can be administered easily and non-invasively is desirable. Thus, PO administration of cochleates will be targeted and higher doses will be tried initially (100 μg/animal and 200 μg/animal). However, lower doses are required for parenteral routes.

Then graded doses will be used to develop a dose response curve for each formulation. Thus, cochleates containing 50 μg, 10 μg, 2 μg, 0.4 and 0 μg polynucleotide/animal will be inoculated with at least 10 animals per group.

Immune response or enzymatic activity are responses easily monitored when expression of the polynucleotide is required. Altered phenotype is another response for tracking efficacy of antisense or ribozyme type molecules. In the case of immune system monitoring, T cell proliferation, CTL and

[redacted]

antibody presence at specific body sites can be valuated, using known m thods, to assess the state of specific immune response.

To determine the duration of activity of cochleate formulations, groups which have responded to a single immunization are monitored periodically for up to a year or more to determine the effective life of a cochleate on administration.

Animals which fail to develop a detectable response on first exposure can be re-inoculated (boosted) to provide insights into the ability of the low dose formulations to prime the immune system for later stimulation.

Pharmaceutical formulations can be of solid form including tablets, capsules, pills, bulk or unit dose powders and granules or of liquid form including solutions, fluid emulsions, fluid suspensions, semisolids and the like. In addition to the active ingredient, the formulation would comprise suitable art-recognized diluents, carriers, fillers, binders, emulsifiers, surfactants, water-soluble vehicles, buffers, solubilizers and preservatives.

An advantage of the cochleates is the stability of the composition. Thus, cochleates can be administered orally, topically or by instillation without concern, as well as by the more traditional routes, such as subcutaneous, intradermal, intramuscular and the like. Direct application to mucosal surfaces is an attractive delivery means made possible with cochleates.

The skilled artisan can determine the most efficacious and therapeutic means for effecting treatment practicing the instant invention. Reference can also be made to any of numerous authorities and references including, for example, "Goodman & Gilman's, The Pharmaceutical Basis for

Therap utics", (6th Ed., Goodman, et al., eds., MacMillan Publ. Co., New York, 1980).

Th cochl ates f the instant inv ntion can be used as a means to transfect cells with an efficacy greater than using currently known delivery means, such as liposomes. Hence, the polynucleotide cochleates of the instant invention provide a superior delivery means for the various avenue of gene therapy, Mulligan, Science 260: 926-931 (1993). As Mulligan noted, the many possibilities of treating disease by gene-based methods will be enhanced by improved methods of gene delivery.

The cochleates of the instant invention also serve as excellent means for delivering other biologically relevant molecules to a host. Such biologically relevant molecules include nutrients, vitamins, co-factors, enzymes and the like. Because the biologically relevant molecule is contained within the cochleate, in a non-aqueous environment, the biologically relevant molecule essentially is stabilized and preserved. As described hereinabove, the biologically relevant molecule is added to the lipid solution and processed to form a precipitated structure comprising lipid and biologically relevant molecule. As demonstrated herein, hydrophilic molecules can be "cochleated", that is, can be made part of the cochleate structure, with little difficulty.

Also, suitable lipophilic biologically relevant molecules, such as drugs and other therapeutic compounds, are amenable to cochleation. For example, lipophilic drugs such as cyclosporin, ivermectin and amphotericin are readily cochleated.

The instant invention now will be described by means of specific examples which are not meant to limit the invention.

EXAMPLE 1

Bovine brain phosphatidylserin in chloroform was purchased from Avanti Polar Lipids, Birmingham, Alabama in glass ampules and stored under nitrogen at -20°C. Cholesterol (porcine liver) grade I, β-D-octyl-glucopyranoside (OCG), fluorescein isothiocyanate (FITC)-dextran (average mol. wt. 67,000), metrizamide grade I, and chemicals for buffers and protein and phosphate determinations, were obtained from Sigma Chemical Company, St. Louis, Missouri. Organic solvents were purchased from Fisher Scientific Co., Fairlawn, New Jersey. Reagents for polyacrylamide gel electrophoresis were from BioRad Laboratories, Richmond, California. S1000 Sephacryl Superfine was obtained from Pharmacia, Piscataway, New Jersey. Thick walled polycarbonate centrifuge tubes (10 ml capacity) from Beckman Instruments, Palo Alto, California, were used for vesicle preparations, washes, and gradients. A bath type sonicator, Model G112SP1G, from Laboratory Supplies Company, Hicksville, New York was used for sonications.

Virus was grown and purified essentially as described by M.C. Hsu et al., Virology, Vol. 95, page 476 (1979). Sendai (parainfluenza type I) and influenza (A/PR8/34) viruses were propagated in the allantoic sac of 10 or 11 day old embryonated chicken eggs. Eggs were inoculated with 1-100 egg infectious doses (10^3 to 10^5 viral particles as determined by HA titer) in 0.1 ml of phosphate buffered saline (0.2 gm/L KCl, 0.2 gm/L KH_2PO_4, 8.0 gm/L NaCl, 1.14 gm/L Na_2H-PO_4, 0.1 gm/L $CaCl_2$, 0.1 gm/L $MgCl_2 6H_2O$ (pH 7.2)). Eggs were incubated at 37°C for 48 to 72 hours, followed by incubation at 4°C for 24 to 48 hours. Allantoic fluid was collected and clarified at 2,000 rpm for 20 minutes

at 5°C in a Damon IEC/PR-J centrifuge. The supernatant was then centrifuged at 13,000 rpm for 60 minutes. This and all subsequent centrifugations were performed in a Sorvall RC2-B centrifuge at 5°C using a GG rotor. The pellets were resuspended in phosphate buffered saline (pH 7.2) by vortexing and sonicating, followed by centrifugation at 5,000 rpm for 20 minutes. The pellet was resuspended by vortexing and sonicating, diluting, and centrifuging again at 5,000 rpm for 20 minutes. The two 5,000 rpm supernatants were combined and centrifuged at 13,000 rpm for 60 minutes. The resulting pellets were resuspended in phosphate-buffered saline by vortexing and sonicating, aliquoted, and stored at -70°C. Sterile technique and materials were used throughout viral inoculation, isolation, and purification.

Virus stored at -70°C was thawed, transferred to sterile thick-walled polycarbonate tubes and diluted with buffer A (2 mM TES, 2 mM L-histidine, 100 mM NaCl (pH 7.4)). Virus was pelleted at 30,000 rpm for 1 hour at 5°C in a Beckman TY65 rotor. The supernatant was removed and the pellet resuspended to a concentration of 2 mg viral protein per ml of extraction buffer (EB) (2 M NaCl, 0.02 M sodium phosphate buffer (pH 7.4)) by vortexing and sonicating. The nonionic detergent β-D-octyl-glucopyranoside was then added to a concentration of 2% (w/v). The suspension was mixed, sonicated for 5 seconds and placed in a 37°C water bath for 45 minutes. At 15, 30 and 45 minute incubation times, the suspension was removed briefly for mixing and sonication. Nucleocapsids were pelleted by centrifugation at 30,000 rpm for 45 minutes in a TY65 rotor. The resulting clear supernatant was removed and used in the formation

of viral glycoprotein-containing cochleat s. Som modification of the abov procedur may have to be mployed with other membrane prot ins. Such modifications are well known to those skilled in the art.

EXAMPLE 2

A. DC Cochleates.

An amount of phosphatidylserine and cholesterol (9:1 wt ratio) in extraction buffer and non-ionic detergent as described hereinabove was mixed with a pre-selected concentration of polynucleotide and the solution was vortexed for 5 minutes. The clear, colorless solution which resulted was dialyzed at room temperature against three changes (minimum 4 hours per change) of buffer A (2 mM TES N-Tris[hydroxymethyl]-methyl-2 aminoethane sulfonic acid, 2 mM L-histidine, 100 mM NaCl, pH 7.4, also identified as TES buffer) containing 3 mM $CaCl_2$. The final dialysis routinely used is 6 mM Ca^{2+}, although 3 mM Ca^{2+} is sufficient and other concentrations may be compatible with cochleate formation. The ratio of dialyzate to buffer for each change was a minimum of 1:100. The resulting white calcium-phospholipid precipitates have been termed DC cochleates. When examined by light microscopy (x 1000, phase contrast, oil), the suspension contains numerous particulate structures up to several microns in diameter, as well as needle-like structures.

B. LC Cochleates.

An amount of phosphatidylserine and cholesterol (9:1 wt ratio) in extraction buffer and non-ionic detergent as described hereinabove was mixed with a pre-selected concentration of

polynucleotid and th solution was vortex d for 5 minut s. Th solution first was dialyzed ov rnight using a maximum ratio of 1:200 (v/v) of dialysate to buffer A without divalent cations, followed by three additional changes of buffer leading to the formation of small protein lipid vesicles. The vesicles were converted to a cochleate precipitate, either by the direct addition of Ca^{2+} ions, or by dialysis against two changes of buffer A containing 3 mM Ca^{2+} ions, followed by one containing buffer A with 6 mM Ca^{2+}.

EXAMPLE 3

IMMUNE RESPONSES TO ORALLY DELIVERED PROTEIN-COCHLEATE VACCINES

To make the vaccine, influenza virus was grown, purified, and the glycoproteins and lipids extracted and isolated as described in Example 1. Protein-cochleates were made according to the "LC cochleate" procedure described above.

Cochleate vaccines containing the glycoproteins and lipids from the envelope of influenza virus and phosphatidylserine and cholesterol were given to mice by gradually dispensing 0.1 ml liquid into the mouth and allowing it to be comfortably swallowed. Figures 4(A) (from Experiment A) and 4(B) (from Experiment B) show resulting total circulating antibody levels specific for influenza glycoproteins, as determined by ELISA. Antibody titer is defined as the highest dilution that still gives the optimal density of the negative control.

In Experiment A that generated the data shown in Figure 4(A), initial vaccine doses of 50, 25, 12.5 or 6.25 μg of glycoproteins (groups 1 through 4 respectively) were administered at 0 and 3 weeks.

The third and fourth immunizations (6 and 19 weeks) were at one fourth the dos used for the initial two immunizations. Bl ed 1 - Bleed 6 occurred at 0, 3, 6, 9, 19, and 21 weeks. The data demonstrate that high circulating antibody titers can be achieved by simply drinking cochleate vaccines containing viral glycoproteins. The response is boostable, increasing with repeated administration, and is directly related to the amount of glycoprotein in the vaccine.

Those observations were confirmed and extended in Experiment B that generated the data shown in Figure 4(B). The dose range was expanded to include 100 μg and 3.1 μg initial doses. Vaccine was given at 0, 3 and 15 weeks, with the third immunization at one fourth the dose of the initial two. Bleed 1 to Bleed 6 occurred at 0, 3, 6, 15 and 16 weeks. Circulating influenza glycoprotein-specific responses were detectable after a single administration for the top five doses, and for all groups after two feedings. The data shown is for pooled sera from each group, but all mice given the four highest doses, and four of five mice in groups five and six, responded to the vaccine with circulating antibody titers ranging from 100 to 102,400. Group seven, which received no vaccine, had titers less than 50 for all mice at all time points.

The antibody response is long lived. Titers 13 weeks after the third immunization (Figure 4(A), bleed 5) and 12 weeks after the second immunization (Figure 4(B), bleed 4) remained the same or within one dilution higher or lower than seen at 3 weeks after the previous boost.

To determine whether oral administration of the subunit vaccine described in Example 2 could lead to protective immunity in the respiratory

tract, the mice described in Experiment B of Example 2 were immunized with cochleates at 0, 3 and 15 weeks. The immunized mice were challenged by intranasal application of 2.5×10^9 particles of influenza virus at 16 weeks. Three days after viral challenge, mice were sacrificed, and lungs and trachea were obtained. The entire lung or trachea was triturated and sonicated, and aliquots were injected into embryonated chicken eggs to allow amplification of any virus present. After three days at 37°C, allantoic fluid was obtained from individual eggs and hemagglutination (HA) titers were performed.

Mice were also challenged with live influenza intranasally following oral cochleate administration in Experiment A of Example 2. Lungs were obtained three days later and cultured to detect presence of virus.

The combined data for the two experiments is given in Table 1. The results also are shown graphically in Figure 5.

TABLE 1

Vaccine Dose μg Protein	Trachea[1] # Infected/Total	Lungs[2] # Infected/Total	Lungs[3] # Infected/Total
100	0/5	0/5	0/5
50	2/5	0/5	2/10
25	0/5	0/5	1/10
125	1/5	0/5	1/10
6.25	0/5	5/5	6/10
3.12	4/5	5/5	5/5
0	5/5	5/5	9/10

1. Mice from Experiment B.
2. Mice from Experiment B.
3. Mice from Experiments A and B.

The data in Table 1 shows that all five of the unvaccinated mice had sufficient virus in the trachea to infect the embryonated chicken eggs (greater than 10^3 particles per trachea or at least one egg infectious dose (EID) per 0.1 ml of suspension). In contrast, the oral vaccine provided a high degree of protection from viral replication in the trachea. All mice in groups 1, 3 and 5 of Experiment B were negative for virus. Two mice in group 2, 1 in group 4, and 4 in group 6 (the lowest vaccine dose) of Experiment B had sufficient virus to test positive in this very sensitive assay used to detect presence of virus.

The oral protein cochleate vaccine also provided protection against viral replication in the lungs. All twenty mice which received the four highest doses of vaccine were negative for virus when lung suspensions were cultured in embryonated chicken eggs (Table 1). All mice in the groups immunized with 6.25 μg and 3.1 μg glycoproteins and

all mice in th unvaccinated control were positive for virus.

Even in the low st two vaccine doses, there was some inhibition of viral replication. When lung suspensions were diluted 1/10 and inoculated into eggs, only one animal in the groups immunized with 6.25 μg was positive, as compared to three in the groups immunized with 3.12 μg and three in the unvaccinated control. Culturing of 1/100 dilutions resulted in one positive animal in each of the groups immunized with 6.25 and 3.12 μg, but 3 of 5 remained positive in the unvaccinated group. In addition, for the two animals in the group that was immunized with 3.12 μg, but which were negative at 1/100, only 50% of the eggs were infected at 1/10 and had low HA titers. In contrast, for the unvaccinated group, all eggs were infected and produced maximal amounts of virus at 1/10 and 1/100 dilutions.

C57BL/6 mice were given cochleates containing Sendai virus glycoproteins orally at 0 and 3 weeks. They were bled at 0 (bleed 1), 3 (bleed 2), and 6 (bleed 3) weeks. Group 1 received approximately 50 μg protein, Group 2 about 25 μg, Group 3 about 12.5 μg, Group 4 about 6.25 μg, and Group 5 (negative control) received 0 μg protein. The levels of Sendai specific antibodies in the serum pooled from 5 mice in each dose group were determined by ELISA. The results are shown in Figure 6. It can be seen that strong antibody responses were generated, that the magnitude of the response was directly related to the immunizing dose, and that the magnitude of the response increased (boosted) after a second immunization.

The response was extremely long-lived. The response is predominantly IgG, indicative of the involvement in T cell help and establishment of

long-term memory cells associated with a secondary immun response. Surprisingly, th lowest dose which initially had the lowest resp nse, n w had the highest circulating antibody levels. This may be due to the immune system's down regulation of the very high responses originally but allowing the low response to slowly climb. This may also indicate a persistence and slow release of antigen. It is also interesting and consistent with the use of the oral route of immunization that significant IgA titers are generated and maintained.

A 50 μg protein dose of Sendai glycoprotein-containing cochleates was given orally. Two weeks later the animal (BALB/c mouse) was sacrificed and spleen cells obtained. Cytolytic activity of the spleen cells was measured by their ability to cause the release of chromium-51 from target cells presenting Sendai antigens. The non-immunized mouse did not kill Sendai virus (SV) pulsed cells with in culture restimulation (N/SV/SV) or non-Sendai presenting cells (N/N/N). (Figure 7) In contrast, Sendai cochleate immunized mice killed SV pulsed targets to a very high degree and non-pulsed targets to a lesser degree. Cytolytic activity is crucial to clearance of cells infected with viruses, or intracellular parasites or to cancer cells. It is a highly desirable activity for a vaccine to induce, but classically has not been seen with most non-living vaccines. This is an important feature of protein-cochleate vaccines.

EXAMPLE 4

Eight week old BALB/c female mice were immunized IM twice with various polynucleotide-cochleate formulations,

polynucleotide alone and controls and then splenocytes from th mic were tested for the ability to proliferate in r sponse to a protein encoded by the polynucleotide.

Cochleates with and without fusogenic Sendai virus protein were prepared as described hereinabove. The polynucleotide used was the pCMVHIVLenv plasmid. The solution containing lipid and extracted Sendai virus envelop proteins as described hereinabove and polynucleotide were mixed at a 10:1 (w/w) ratio and 50:1 (w/w) ratio. That protocol yielded four groups, cochleate/DNA, 10:1; cochleate/DNA, 50:1; SV-cochleate/DNA, 10:1; and SV-cochleate/DNA, 50:1. Naked DNA was used at a rate of 10 μg/mouse and 50 μg/mouse. The control was buffer alone. Mice were immunized twice, 15 days apart at 50 μl/mouse.

Splenocytes were obtained and tested in a T-cell proliferation assay using tritiated thymidine, as known in the art. Control cultures contained no antigen or con A. The antigen used was p18 peptide, at 1 μM, 3 μM and 6 μM. Cells were harvested at days 2, 4 and 6 following preparation of the splenocyte cultures.

The naked DNA provided a marginal response above background. All four cochleate preparations yielded a p18-specific response which increased over time. At six days, the response was about four times above background.

The DNA concentration range at the 10:1 ratio was about 120-170 μg/ml. At the 50:1 (w/w) ratio; the DNA concentration was about 25-35 μg/ml.

The polynucleotide-cochleates were exposed to micrococcal nuclease and little or no nucleic acid degradation was observed.

The polynucleotide encapsulation efficiency was found to be about 50% based on quantification

of free DNA from lipid, that is present in the supernatant following a precipitation reaction. After washing th precipitate and opening the structures by removing cation about 35% of the DNA was recovered.

EXAMPLE 5

In similar fashion, splenocytes from animals immunized as described in Example 4, were tested for antigen specific cytotoxic activity using a chromium release assay using labelled H-2 compatible target cells known to express an HIV protein, such as gp160. The responder cells can be stimulated by brief exposure to purified HIV peptides.

On prestimulation, animals exposed to polynucleotide cochleates demonstrated specific cytotoxic splenocytes directed to gp160, with nearly 100% cytotoxicity observed at an effector:target ratio of 100.

EXAMPLE 6

Fifteen mg of insulin were added to 15 ml of extraction buffer (EB) in a 50 ml plastic tube. Then 300 mg of OCG were added to the mixture. The resulting suspension was colloidal and not clear at pH 7.4. The solution was titrated with 1 N NaOH to pH 8.5, resulting in a clear solution.

In a separate vessel, 6.8 ml of a 10 mg/ml solution of phosphatidylserine and 1.5 ml of a 5 mg/ml solution of cholesterol were mixed and then dried to yield a thin film. The insulin solution was added to the vessel yielding a colloidal suspension. The suspension was vortexed for seven minutes and then set on ice for one hour. The pH

of th solution was adjust d to 9-9.5 with 1 N NaOH, th sample was filter sterilized and placed in dialysis tubing at about 2 ml per bag.

Two different dialysis schedules were used.

A. DC cochleates:

1. 100 ml overnight 1 x TES pH 9.0 containing 3 mM Ca^{+2}, Zn^{+2} or Mg^{+2}

2. 250 ml 4h 1 x TES pH 8.5 containing 3 mM Ca^{+2}, Zn^{+2} or Mg^{+2}

3. 250 ml 4h 1 x TES pH 8.0 containing 3 mM Ca^{+2}, Zn^{+2} or Mg^{+2}

4. 250 ml 4h 1xTES pH 7.4 containing 6 mM Ca^{+2}, Zn^{+2} or Mg^{+2}

B. LC cochleates:

1. 100 ml overnight 1 x TES, pH 9.0

2. 250 ml 4h, 1 x TES, pH 9.0

3. 250 ml 4h 1 x TES, pH 9.0

4. 100 ml overnight 1 x TES, pH 9.0 containing 3 mM Ca^{+2}, Zn^{+2} or Mg^{+2}

5. 250 ml 4h 1 x TES, pH 8.5 containing 3 mM Ca^{+2}, Zn^{+2} or Mg^{+2}

6. 250 ml 4h 1 x TES, pH 7.4 containing 6 mM Ca^{+2}, Zn^{+2} or Mg^{+2}

Following dialysis, the resulting precipitate was found to comprise numerous cochleates.

EXAMPLE 7

Mice were given insulin cochleate samples orally. Serum glucose levels were measured at 0 time, (prior to cochleate administration), 30 min. and 60 min. post administration using standard methods. Cochleate formulations of Example 6 with

a starting concentration of 1 mg insulin/ml solution were used. Each mouse was administered 100 ul or 200 ul of the designated preparations as indicated. For comparison, one mouse was given the standard commercial human insulin, Humulin R, by intraperitoneal administration.

Sample	Volume Given	Serum Glucose mg/dl		
		0 Time	30 min.	60 min.
LC Ca++	200 ul	100	49.12	43
LC Ca++	200 ul	102.9	252.4	61.9
Humulin R	200 ul	88.8	66	48.5

Oral administration of insulin affected serum glucose levels.

EXAMPLE 8

Insulin cochleates as produced in Example 6 were fed orally to three-month-old female BALB/c mice made diabetic through intraperitoneal injection of streptozotocin, practicing known methods. Two days after exposure to streptozotocin, the mice were allocated into groups of five and administered with oral insulin cochleates at 200 ρl per mouse. Other mice were injected with 2 IU of Humulin R.

Serum samples were obtained at time 0, prior to insulin dosing, and two hours post insulin administration. Glucose levels were measured using a kit from Sigma (St. Louis). Control animals were untreated, that is, received no streptozotocin or insulin. Representative data are set forth in Figure 8. Orally administered insulin, simply by

drinking, was effective in reducing blood glucose levels. No reduction in blood glucose was obs rved in control animals.

All references cited herein are incorporated by reference in entirety.

While the invention has been described in detail and with reference to specific embodiments thereof, it will be apparent to one skilled in the art that various changes and modifications can be made therein without departing from the spirit and scope thereof.

What is claimed is:

1. A formulati n comprising

a) a polynucleotide or polypeptide component;

b) a negatively charged lipid component, and

c) a divalent cation component.

2. The formulation of claim 1, wherein said polynucleotide component is deoxyribonucleic acid.

3. The formulation of claim 2, wherein said deoxyribonucleic acid is transcribed to yield a ribonucleic acid.

4. The formulation of claim 3, wherein said ribonucleic acid is translated to yield a polypeptide.

5. The formulation of claim 1, wherein said polynucleotide component is ribonucleic acid.

6. The formulation of claim 1 wherein said polypeptide is a hormone.

7. The formulation of claim 6, wherein said hormone is insulin.

8. The formulation of claim 1, wherein said lipid component is phospholipid.

9. The formulation of claim 8, wherein the phospholipid is selected from the group consisting of phosphatidylserine, phosphatidylglycerol, phosphatidylinositol, and phosphatidic acid.

10. The formulation of claim 1, wherein the divalent cation component is a cationic compound capabl f chelating and complexing negatively charged lipids.

11. The formulation of claim 10, wherein the divalent cation component is selected from the group consisting of Ca^{+2}, Mg^{+2}, Ba^{+2} and Zn^{+2}.

12. The formulation of claim 11, wherein the divalent cation component is Ca^{+2}.

13. A method of administering a biologically relevant molecule to a cell in a host comprising administering a biologically effective amount of a cochleate formulation comprising

a) a biologically relevant molecule component;

b) a negatively charged lipid component, and

c) a divalent cation component.

14. The method of claim 13, wherein said biologically relevant molecule is a polynucleotide.

15. The method of claim 13 wherein said biologically relevant molecule is a polypeptide.

16. The method of claim 15 wherein said polypeptide is insulin.

17. The method of claim 13, wherein said biologically relevant molecule is a lipophilic drug.

18. The method of claim 17, wherein said lipophilic drug is selected from the group

consisting of cyclosporin, ivermectin and amphotericin.

19. A timed release formulation comprising a plurality of species of cochleates, wherein said said species of cochleates comprise at least two divalent cations.

20. The timed release formulation of claim 19, wherein said species of cochleates comprise a plurality of biologically relevant molecules.

21. The timed release formulation of claim 19, wherein said species of cochleates comprise a biologically relevant molecule.

22. The timed release formulation of claim 19, wherein said biologically relevant molecule is selected from the group consisting of a non-peptide hormone, a non-peptide drugs and a polypeptide.

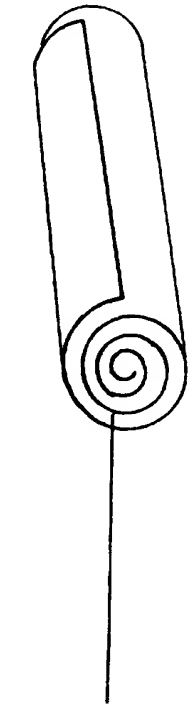
23. The timed release formulation of claim 22, wherein said polypeptide is selected from the group consisting of hormones, cytokines and enzymes.

24. The timed release formulation of claim 23, wherein said polypeptide is a hormone.

25. The timed release formulation of claim 24, wherein said hormone is insulin.

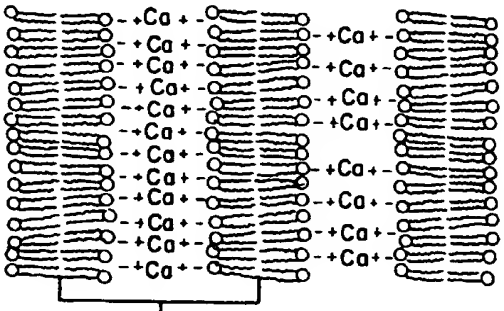
26. The timed release formulation of claim 22, wherein said drug is selected from the

group consisting of cyclosporin, ivermectin and amphotericin.



COCHLEATE

BILAYERS TIGHTLY APPOSED
53A REPEAT DISTANCE.
APPROXIMATELY 52A OCCUPIED BY EACH LIPID BILAYER THEREFORE ONLY IA BETWEEN BILAYERS.
SINGLE Ca ION BRIDGING.
WATER IS EXCLUDED.



REPEAT DISTANCE
53Å

PHOSPHATIDYLSERINE

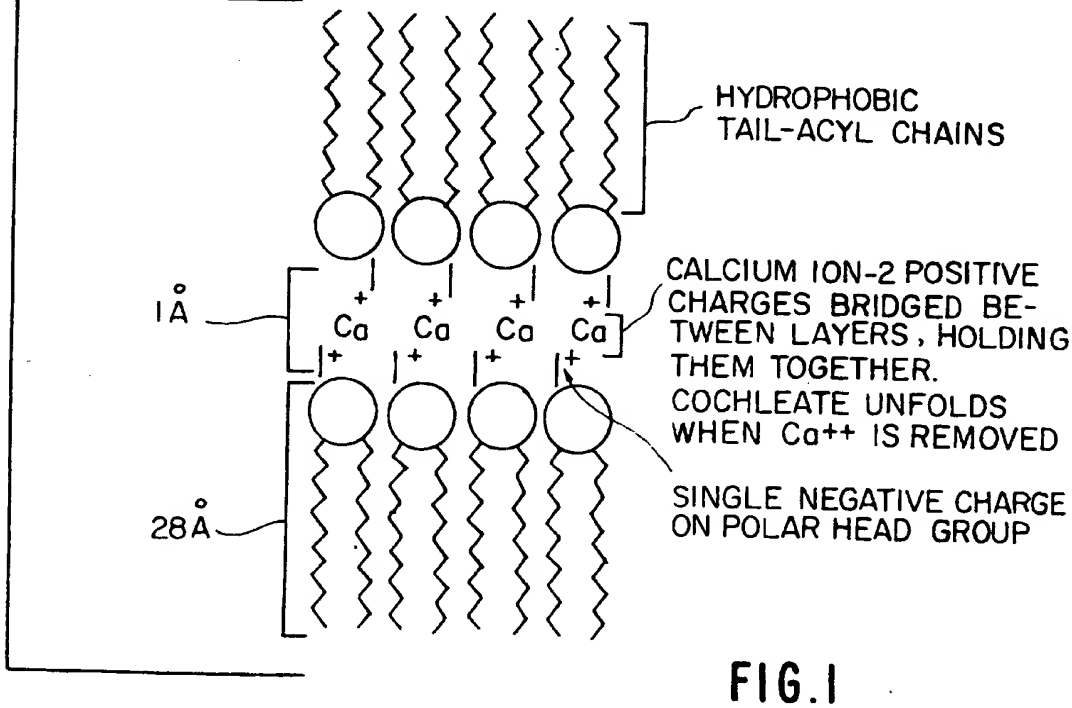


FIG.1

[redacted]

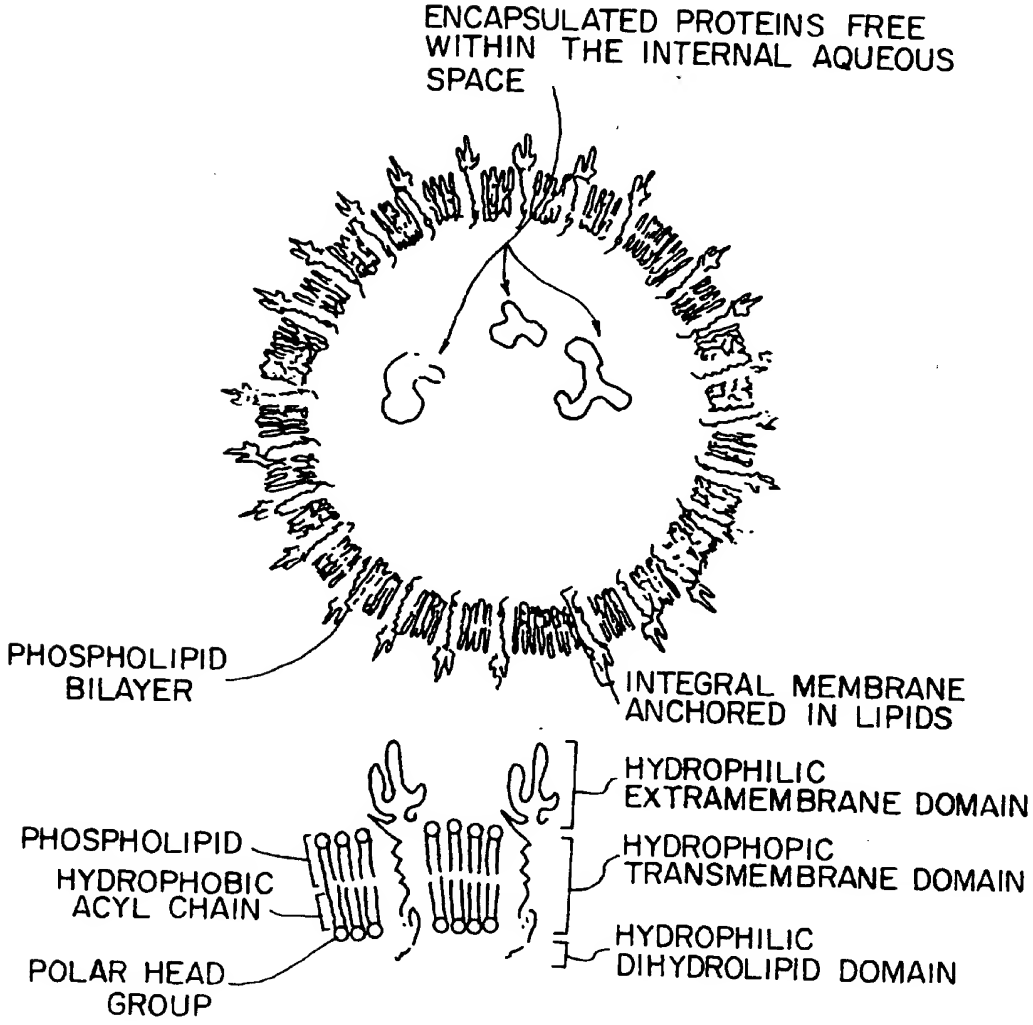

ENCAPSULATED PROTEINS FREE WITHIN THE INTERNAL AQUEOUS SPACE
PHOSPHOLIPID BILAYER
INTEGRAL MEMBRANE ANCHORED IN LIPIDS
HYDROPHILIC EXTRAMEMBRANE DOMAIN
HYDROPHOPIC TRANSMEMBRANE DOMAIN
HYDROPHILIC DIHYDROLIPID DOMAIN
PHOSPHOLIPID
HYDROPHOBIC ACYL CHAIN
POLAR HEAD GROUP

FIG. 2

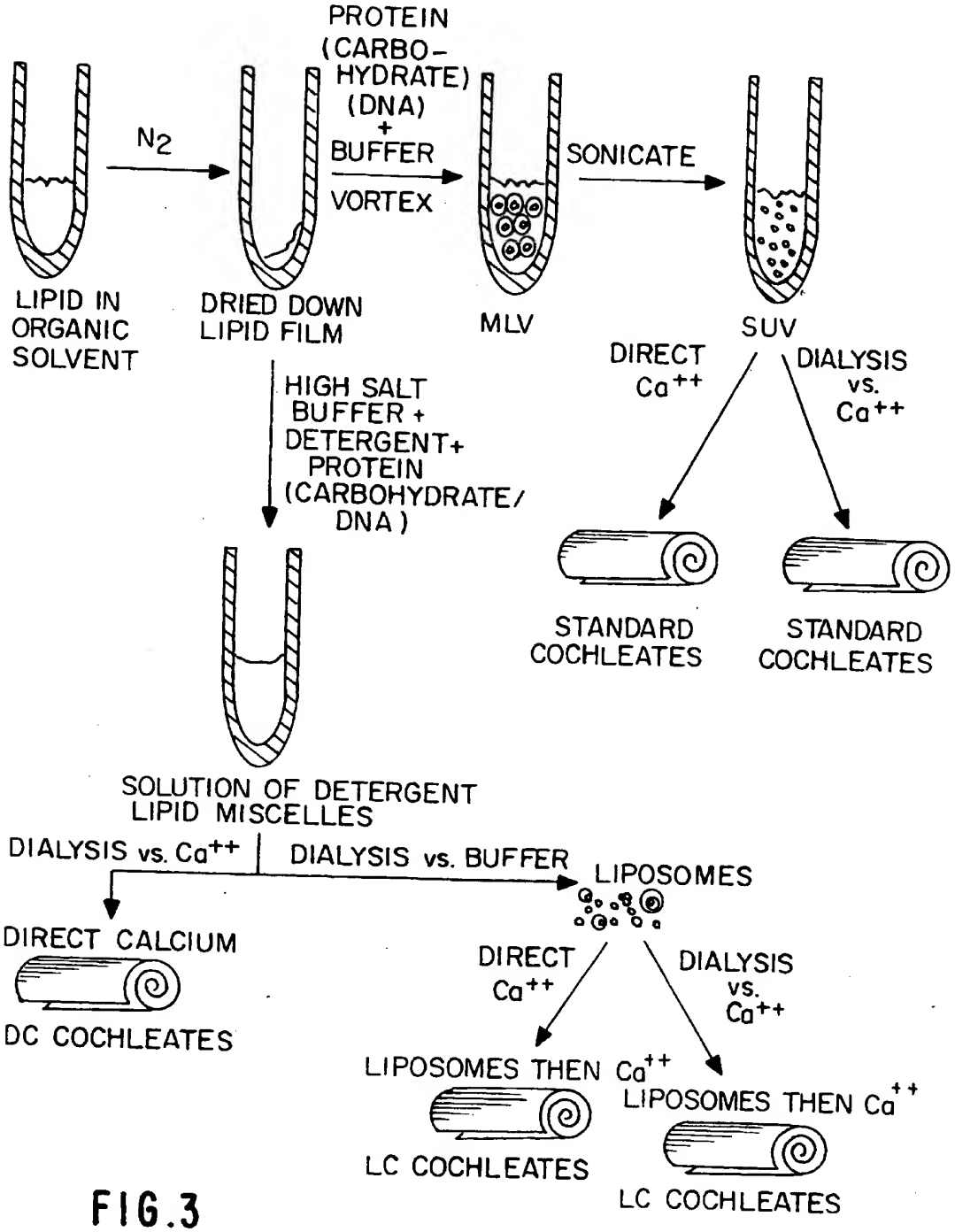

PROTEIN
(CARBO-
HYDRATE)
(DNA)
+
BUFFER
VORTEX
N2
SONICATE
LIPID IN
ORGANIC
SOLVENT
DRIED DOWN
LIPID FILM
MLV
SUV
DIRECT
Ca++
DIALYSIS
vs.
Ca++
HIGH SALT
BUFFER +
DETERGENT+
PROTEIN
(CARBOHYDRATE/
DNA)
STANDARD
COCHLEATES
STANDARD
COCHLEATES
SOLUTION OF DETERGENT
LIPID MISCELLES
DIALYSIS vs. Ca++
DIALYSIS vs. BUFFER
LIPOSOMES
DIRECT CALCIUM
DC COCHLEATES
DIRECT
Ca++
DIALYSIS
vs.
Ca++
LIPOSOMES THEN Ca++
LIPOSOMES THEN Ca++
LC COCHLEATES
LC COCHLEATES

FIG.3

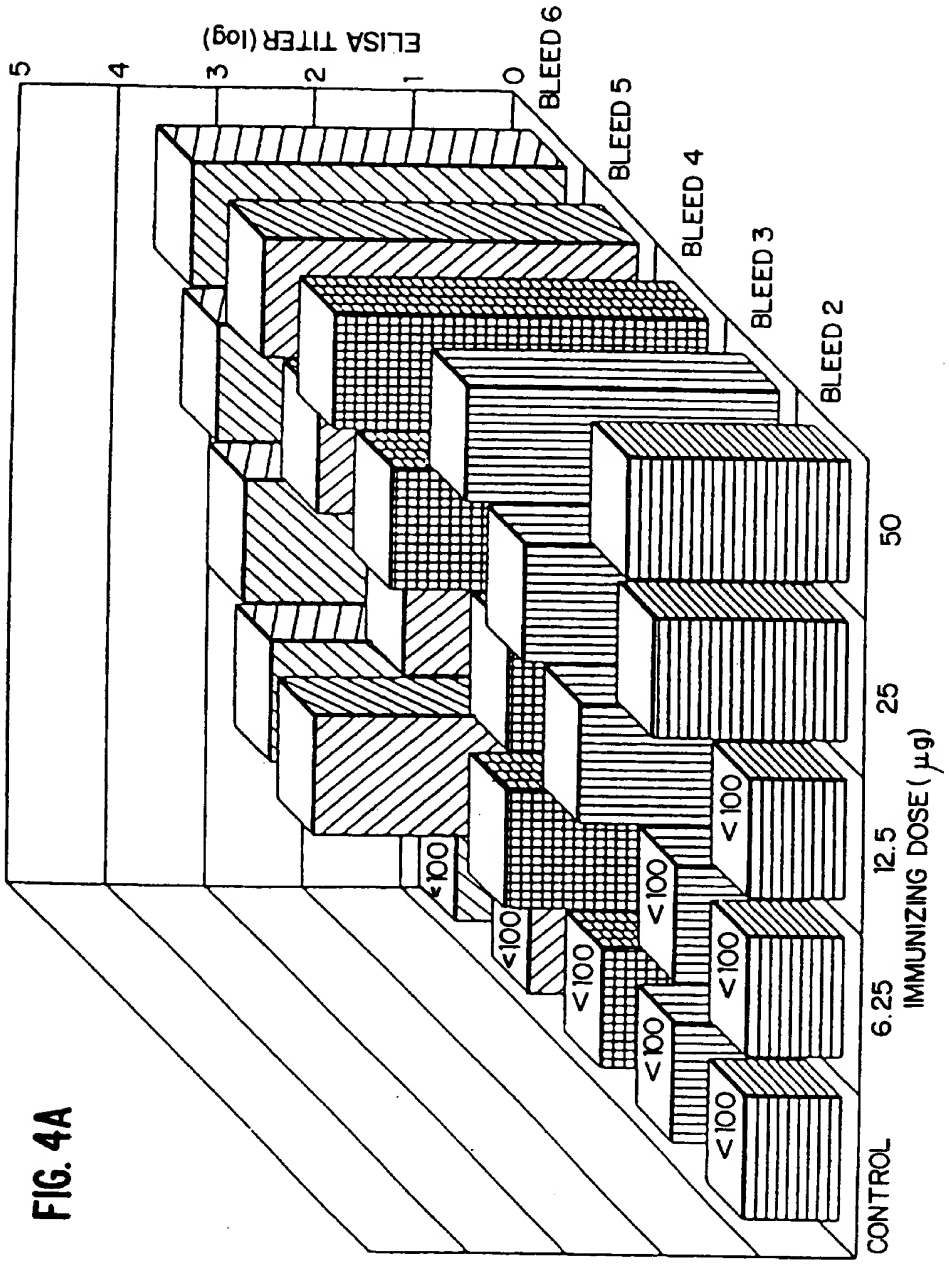
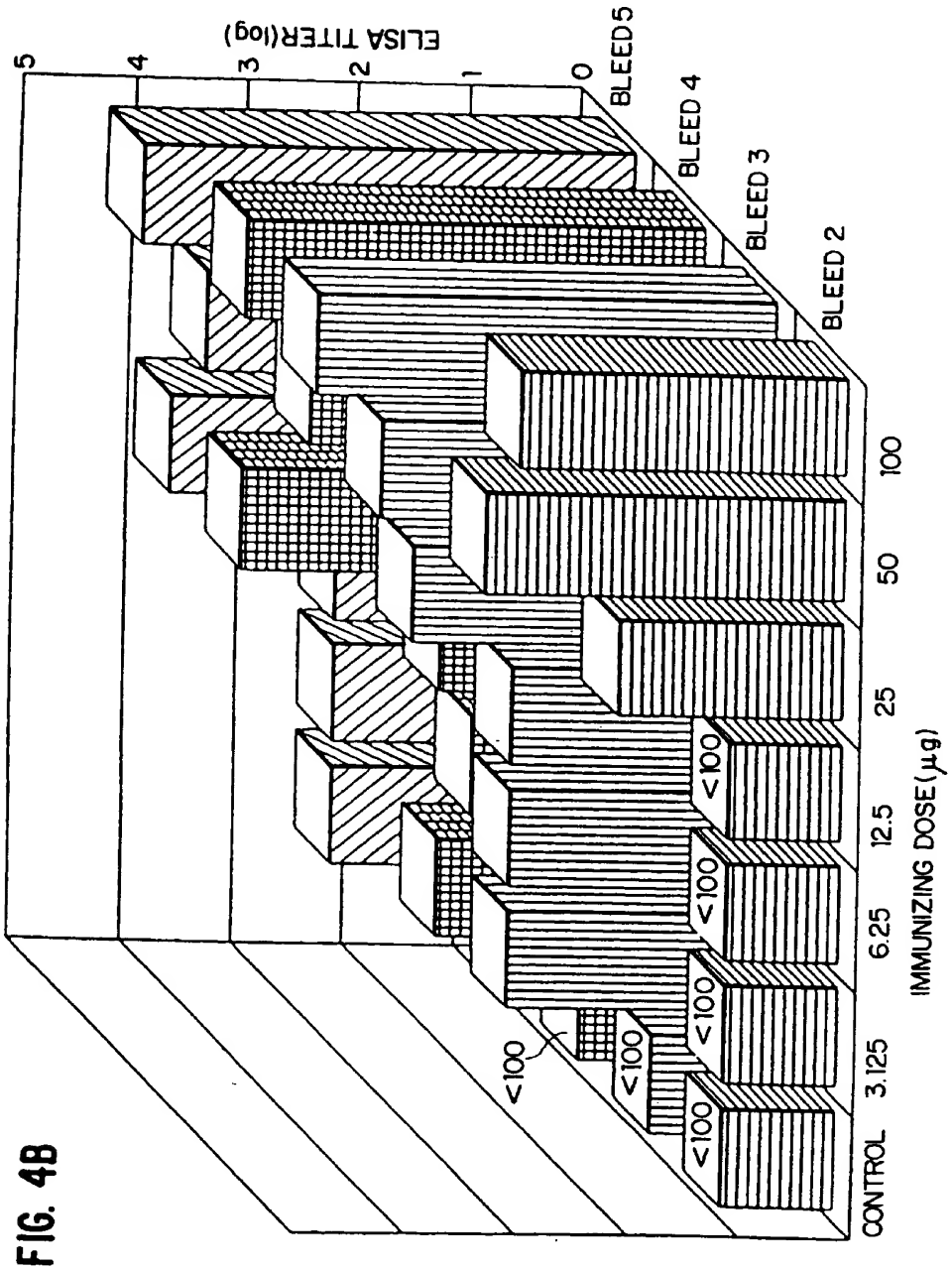

ELISA TITER (log)
5
4
3
2
1
0
BLEED 6
BLEED 5
BLEED 4
BLEED 3
BLEED 2
CONTROL
6.25
12.5
25
50
IMMUNIZING DOSE (μg)
<100

FIG. 4A


ELISA TITER(log)
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4
3
2
1
0
BLEED 5
BLEED 4
BLEED 3
BLEED 2
<100
<100
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<100
CONTROL
3.125
6.25
12.5
25
50
100
IMMUNIZING DOSE(μg)

FIG. 4B

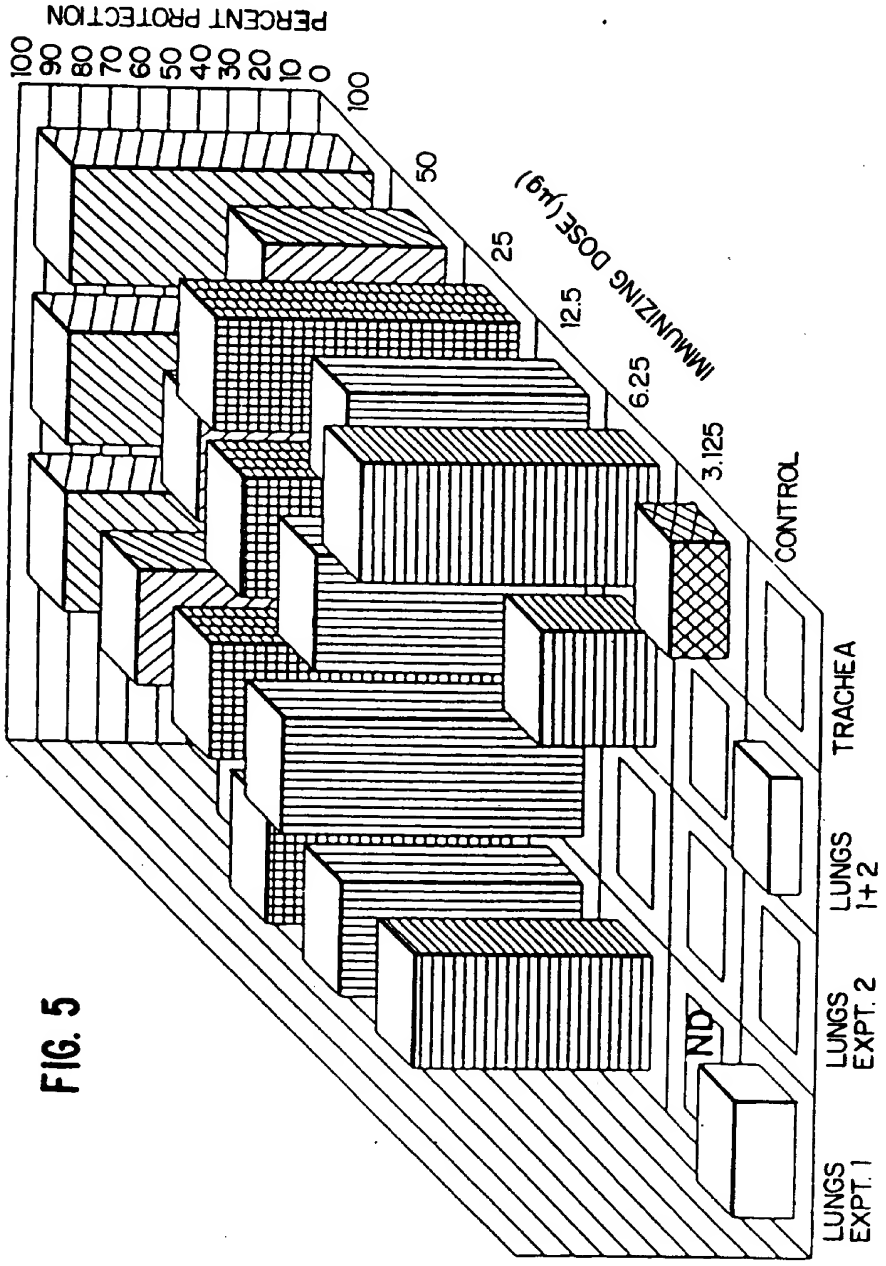

PERCENT PROTECTION
100
90
80
70
60
50
40
30
20
10
0
IMMUNIZING DOSE (μg)
100
50
25
12.5
6.25
3.125
CONTROL
TRACHEA
LUNGS 1+2
LUNGS EXPT. 2
ND
LUNGS EXPT. 1

FIG. 5

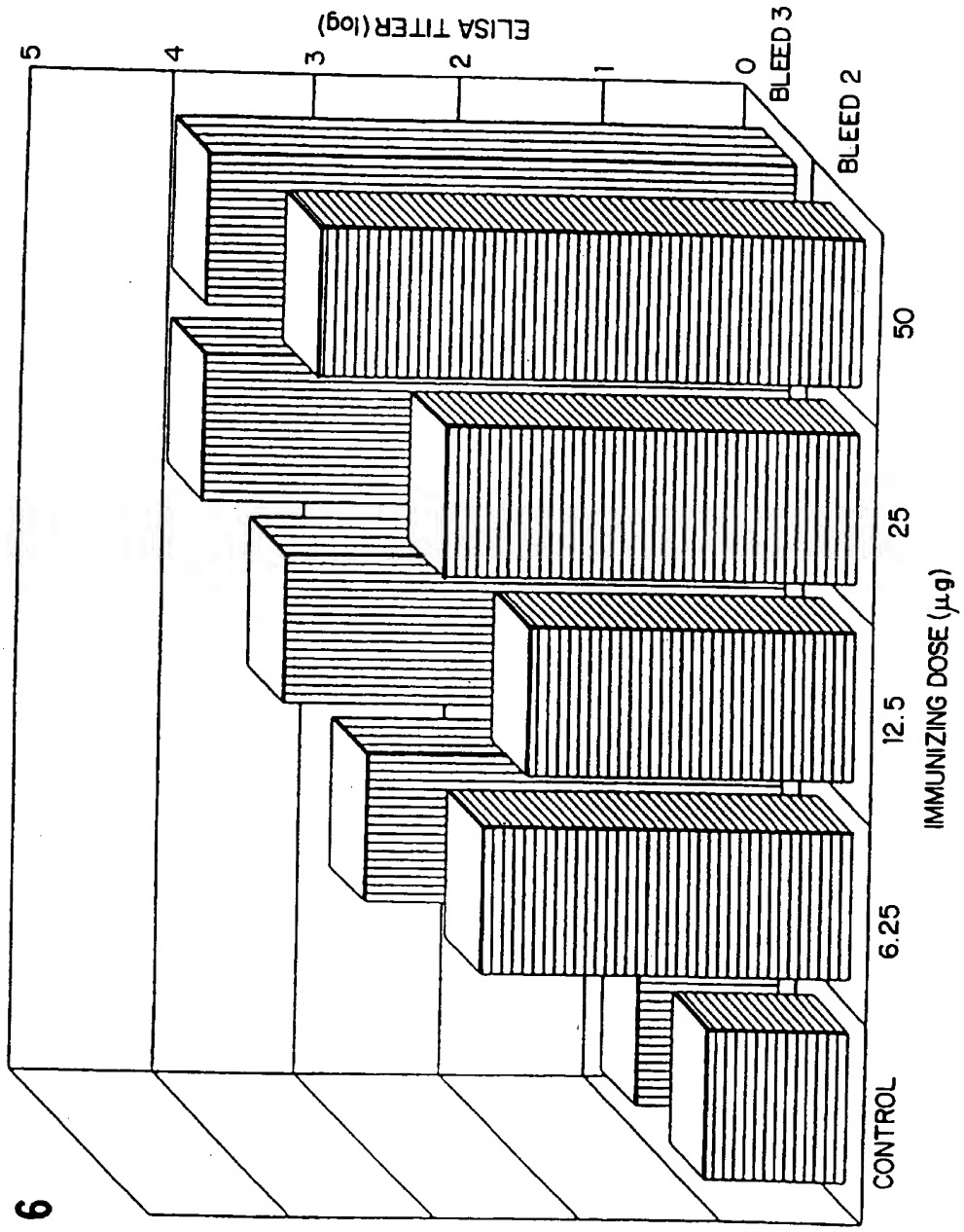

ELISA TITER (log)
5
4
3
2
1
0
BLEED 3
BLEED 2
50
25
12.5
6.25
CONTROL
IMMUNIZING DOSE (μg)

FIG. 6

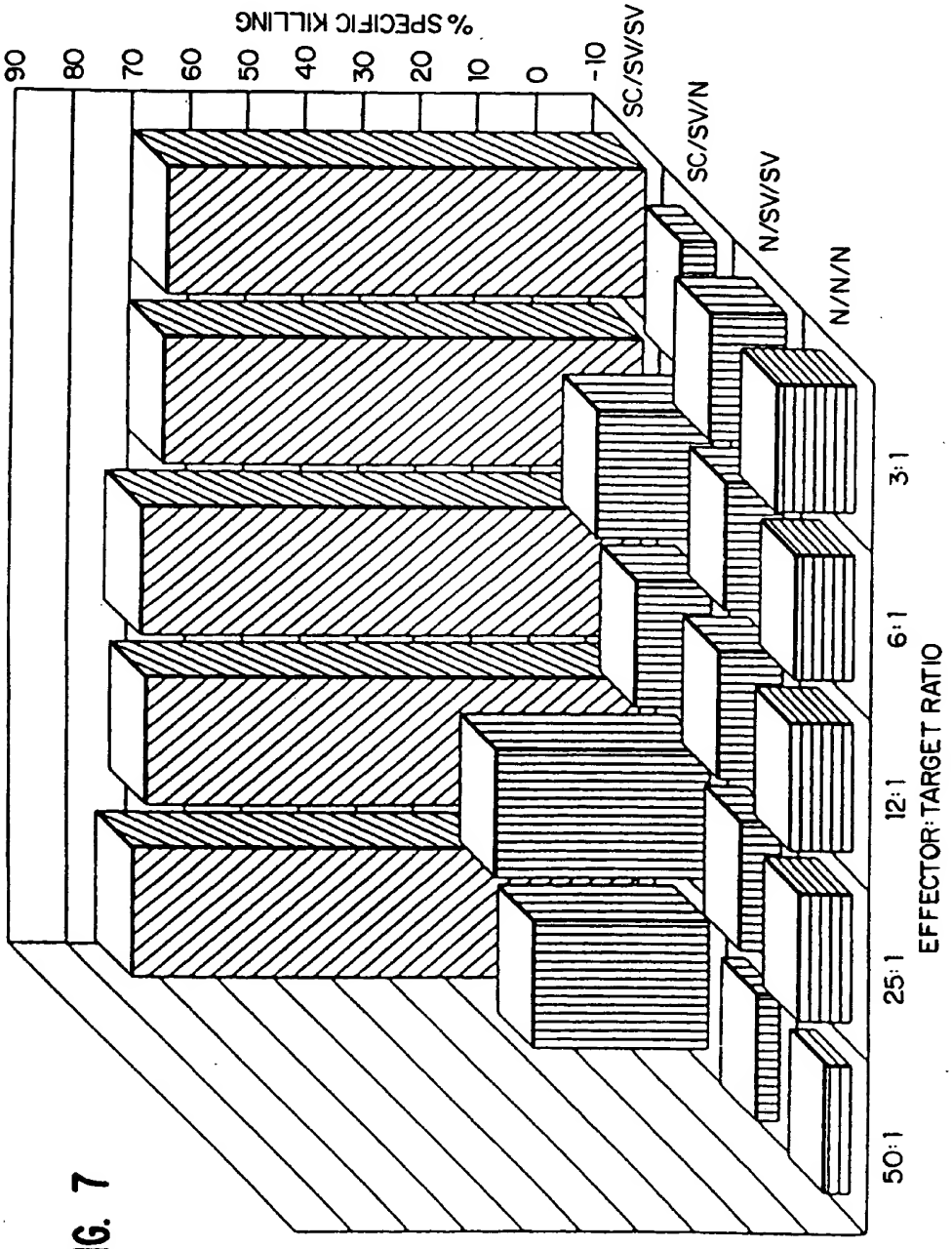
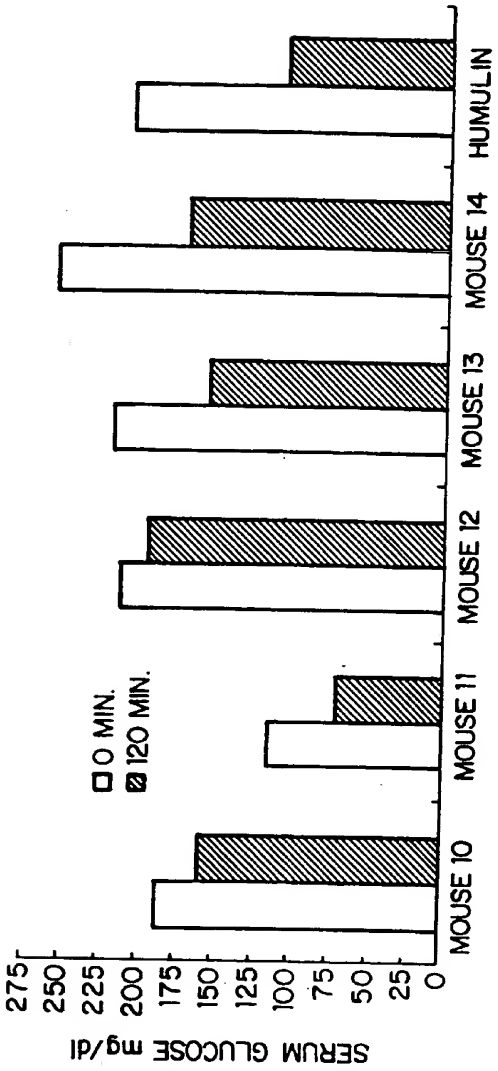

% SPECIFIC KILLING
90
80
70
60
50
40
30
20
10
0
-10
SC/SV/SV
SC/SV/N
N/SV/SV
N/N/N
50:1
25:1
12:1
6:1
3:1
EFFECTOR:TARGET RATIO

FIG. 7

FIG. 8


SERUM GLUCOSE mg/dl
275
250
225
200
175
150
125
100
75
50
25
0
0 MIN.
120 MIN.
MOUSE 10
MOUSE 11
MOUSE 12
MOUSE 13
MOUSE 14
HUMULIN

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/01704

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A61K 38/28, 31/70

US CL :264/4.1; 424/1.21; 426/829; 428/402.24; 514/3, 44

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 264/4.1; 424/1.21; 426/829; 428/402.24; 514/3, 44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ---- Y	US 4,663,161 A (R.J. MANNINO ET AL.) 05 May 1987, column 3,lines 29-61; column 12, lines 40-45.	1, 8, 9-12 ------ 2-6
X	US 4,871,488 A (R.J. MANNINO ET AL.) 03 October 1989, column 3, lines 33-65.	1, 8-12
X ---- Y	US 4,078,052 A (P.D. PAPAHADJOPOULOS) 07 March 1978, column 2, line 36 to column 3, line 40; column 3, lines 43-65.	1, 8-12 ---- 13-15
Y ---- Y	MORI et al. Immunotargeting of liposomes containing lipophilic antitumor prodrugs. Pharmaceutical Research. 1993, Vol. 10, No. 4, pages 507-514, especially page 508 column 1, lines 30-52; page 509 column 1, line 24 to column 2, line 8.	1, 8-12 ---- 17

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 20 MAY 1996	Date of mailing of the international search report 13 JUN 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer PATRICK TWOMEY, PH.D. Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

[redacted]

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/01704

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PAPAHADJOPOULOS et al. Cochleate lipid cylinders: formation by fusion of unilamellar lipid vesicles. Biochimica et Biophysica Acta. 1975, Vol. 394, pages 483-491, see entire document.	1, 8-12
Y	BOOSER et al. Anthracycline antibiotics in cancer therapy. Drugs. 1994, Vol. 47, No. 2, pages 223-258, especially page 228 column 2, line 30 to page 230 column 1, line 2.	1, 8-12, 17
X	GOULD-FOGERITE et al. Chimerasome-mediated gene transfer in vitro and in vivo. Gene. 1989, Vol. 84, pages 429-438, especially page 430 column 2, line 28 to page 431 column 2, line 32; page 433 column 1, line 45 to page 436 column 2, line 22.	1-5, 8-12

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/01704

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

DIALOG MEDICINE AND BIOTECH CLUSTERS
cochleate, liposome, phosphatidylserine, phosphatidylglycine, phosphatidylinositol, hormone, insulin, lipophilic drug, cyclosporin, ivermectin, amphotericin, DNA.
APS
mannino, fogerite, liposome, cochleate, DNA